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## **Interferon-induced antiviral protein MxA interacts with the cellular RNA helicases UAP56 and URH49**

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**Abstract:** Mx proteins are a family of large GTPases that are induced exclusively by interferon-alpha/beta and have a broad antiviral activity against several viruses, including influenza A virus (IAV). Although the antiviral activities of mouse Mx1 and human MxA have been studied extensively, the molecular mechanism of action remains largely unsolved. Because no direct interaction between Mx proteins and IAV proteins or RNA had been demonstrated so far, we addressed the question of whether Mx protein would interact with cellular proteins required for efficient replication of IAV. Immunoprecipitation of MxA revealed its association with two closely related RNA helicases, UAP56 and URH49. UAP56 and its paralog URH49 play an important role in IAV replication and are involved in nuclear export of IAV mRNAs and prevention of dsRNA accumulation in infected cells. In vitro binding assays with purified recombinant proteins revealed that MxA formed a direct complex with the RNA helicases. In addition, recombinant mouse Mx1 was also able to bind to UAP56 or URH49. Furthermore, the complex formation between cytoplasmic MxA and UAP56 or URH49 occurred in the perinuclear region, whereas nuclear Mx1 interacted with UAP56 or URH49 in distinct dots in the nucleus. Taken together, our data reveal that Mx proteins exerting antiviral activity can directly bind to the two cellular DExD/H box RNA helicases UAP56 and URH49. Moreover, the observed subcellular localization of the Mx-RNA helicase complexes coincides with the subcellular localization, where human MxA and mouse Mx1 proteins act antivirally. On the basis of these data, we propose that Mx proteins exert their antiviral activity against IAV by interfering with the function of the RNA helicases UAP56 and URH49.

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**The Interferon Induced Antiviral Protein MxA interacts with The Cellular RNA Helicases  
UAP56 and URH49**

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Running head: MxA interacts with the cellular helicase UAP56

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**Mx proteins are a family of large GTPases which are exclusively induced by interferon- $\alpha/\beta$  and have a broad antiviral activity against several viruses, including influenza A virus (IAV). Although the antiviral activity of the mouse Mx1 and the human MxA have been studied extensively, the molecular mechanism of action remains largely unsolved. Since no direct interaction between Mx proteins and IAV proteins or RNA was demonstrated so far, we addressed the question whether Mx protein would interact with cellular proteins required for efficient replication of IAV. Immunoprecipitation of MxA revealed its association with two closely related RNA helicases UAP56 and URH49. UAP56 and its paralog URH49 play an important role for IAV replication and are involved in nuclear export of IAV mRNAs and prevention of dsRNA accumulation in infected cells. In vitro binding assays with purified recombinant proteins revealed that MxA forms a direct complex with the RNA helicases. In addition, recombinant mouse Mx1 was also able to bind to UAP56 or URH49. Furthermore, the complex formation between the cytoplasmic MxA and UAP56 or URH49 occurred in the perinuclear region while the nuclear Mx1 interacted with UAP56 or URH49 in distinct dots in the nucleus. Taken together our data revealed that Mx proteins exerting antiviral activity can directly bind to the two cellular DExD/H-box RNA helicases UAP56 or URH49. Moreover, the observed subcellular localization of the Mx-RNA helicase complexes coincides with the subcellular localization where human MxA and mouse Mx1 proteins act antivirally. Based on these data we propose that Mx proteins exert their antiviral activity against IAV by interfering with the function of the RNA helicases UAP56 and URH49.**

Mx proteins belong to a family of dynamin-like large GTPases. They play a pivotal role in the type I-interferon mediated response against a broad range of viral infections (1). The human MxA protein accumulates in the cytoplasm and has been shown to inhibit several RNA and DNA viruses including influenza A virus (IAV), vesicular stomatitis virus (VSV), thogotovirus (THOV) and La Crosse virus (LACV) (1-3). The murine Mx1 protein is a nuclear protein and inhibits the replication of several members of the orthomyxovirus family, including IAV (1).

Mx proteins share a high intrinsic GTPase activity and the ability to form large oligomeric structures (4). Recently the crystal structure of the stalk region of MxA was solved and in combination with similar structural data from dynamin a model for a four helical bundle formation of MxA was proposed (5). It is not clear whether Mx proteins exert their antiviral activity in form of large oligomeric structures or monomers. Mutations preventing the intermolecular Mx-Mx interactions abrogate the antiviral activity (5), while a monomeric form of MxA with a mutation abolishing the intramolecular backfolding of the carboxy terminal end remains active (6-8). Although the antiviral function of MxA has been studied extensively, little is known about the molecular mechanism of the inhibition. MxA inhibits THOV infection via a physical interaction with the THOV nucleocapsids, blocking the nuclear import of these nucleocapsids (9). For LACV an interaction between MxA and the LACV nucleoprotein was also shown (10). However, for IAV no direct interaction of Mx proteins with any viral protein could be demonstrated. Hence we hypothesized that Mx proteins may interfere with the function of cellular protein(s) required for replication of IAV. Human MxA as well as mouse Mx1 protein have been shown to associate with several cellular proteins of the cytoskeleton or the proteosomal degradation pathway, but none of these proteins appear to

play a role in IAV replication (11,12). We have previously shown that overexpression of the IAV proteins PB2 and also NP (both proteins part of the vRNP complex) partially overcome the inhibition of the mouse Mx1 protein (13,14). More recent studies revealed that the Mx resistant phenotype of certain influenza virus strains segregates with the NP protein (15,16). Therefore, we tested whether cellular proteins known to be functionally relevant for the replication of IAV or known to associate with viral proteins or vRNPs (17,18) would also interact with Mx proteins. For this purpose we expressed the cDNAs of several cellular proteins (kindly provided by Dr. Peter Palese, New York) that were identified to bind to the nucleoprotein of influenza A (18) in cells transfected with human MxA. Preliminary experiments rapidly revealed that only the DExD/H-box RNA helicase Bat-1/UAP56 co-immunoprecipitated with the human MxA protein. UAP56 plays an important role in the assembly of the spliceosome and in nuclear export of spliced and unspliced mRNA out of the nucleus. It has been first described as an essential splicing factor required for spliceosome assembly (19-21). Additionally, UAP56 plays a pivotal role for the nuclear export of mRNA into the cytoplasm (22). Recently, a close paralog of UAP56 termed UAP56-related helicase, 49 kDa (URH49) has been identified that has 90% amino acid identity and exhibits similar cellular functions (23,24). Several reports have demonstrated that UAP56 and URH49 also play a role for efficient replication of IAV (18,25-27). Momose and colleagues have shown that the interaction of NP with UAP56 leads to an increased vRNA synthesis in vitro (18). In addition, UAP56 and URH49 are required for the efficient export of nascent IAV mRNAs (26,27) and it was recently proposed that UAP56 is involved in the encapsidation of viral cRNA with NP (25). Moreover, we recently demonstrated that UAP56 is required for the prevention of dsRNA formation during IAV infection thereby preventing the activation of the interferon type I system (27). In this study we provide evidence that human MxA and mouse Mx1 interact with the cellular helicases UAP56 and URH49. In vitro studies with recombinant proteins reveal that the interaction is direct. Furthermore co-localization experiments clearly indicate that Mx1 and UAP56 or URH49 form a complex as expected in the cell nucleus while the interaction between

MxA and UAP56 or URH49 occurs in the cytoplasm.

## EXPERIMENTAL PROCEDURES

**Cells and transfections** - A549, 3T3 and HEK 293T (ATCC) cells were cultured in DMEM (Gibco) supplemented with 10% FCS, 1% Pen/Strep (Gibco) and 1% Glutamax (Gibco). Cells were transfected at 80% confluency with jetPEI transfection reagent (Polyplus transfection) according to the manual and incubated for 24h before being analyzed.

**Western blotting and Co-immunoprecipitation** - 293T cells were grown in 10cm cell culture dishes (TPP) and transfected at 80% confluency with the indicated amounts of plasmid. 48 hours after transfection cells were lysed in 500µl lysis buffer (0.5% Triton X-100, 20mM TRIS pH 7.5, 100mM NaCl, 50mM β-glycerolphosphate, 50mM sodiumfluoride, 1mM sodiumortho-vanadate and a protease inhibitor cocktail (Roche). Co-immunoprecipitations were performed with 1µg mouse anti-FLAG antibody (SIGMA) for 4 hours at 4°C. Immunoprecipitations were done at room temperature for 1 hour using 50µl protein-G beads (Dynalbeads, Invitrogen). Samples were washed three times with lysis buffer and beads were taken up in 40µl SDS-Laemmli buffer and heated for 5 min to 95°C. Samples were loaded on 10% SDS-gels followed by immunoblot analysis with different antibodies. Anti-FLAG antibody (1:3000, SIGMA) against mouse and rabbit, anti-URH49 (1:750, Acris), anti-UAP56 (serum from a mouse immunized with a peptide of human UAP56), anti-NP mouse monoclonal HB65 (1:3). Primary antibodies were incubated over night at 4°C and incubated the next day for 1 hour with anti-HRP-conjugated secondary antibodies from GE-Healthcare (1:10000). Membranes were analyzed on a Fuji imager using the Multi Gauge 3.0 software.

**Expression constructs and protein purification** - URH49 and UAP56 were cloned into pGEX-3x (GE Healthcare) with an N-terminal GST fusion tag (URH49-for: 5'-GATAAGAATTCCGCAGAACAGGATGTGG AAAACGATC-3' (EcoRI), URH-49-rev: 5'-CTAATGAATTCAATTTACCGGCTCTGCTC GATGTATGTG-3' (EcoRI), UAP56-for: 5'-CTTATACCCGGGGCAGAGAACGATGTGG ACAATG-3' (SmaI), UAP56-rev: 5'-CAATAATCCCGGGGATAAACTACCGTGTC TGTTCATGTA-3' (SmaI). Proteins were

expressed in E.coli BL21 with 0.1mM IPTG for 4 hours at 32°C. Bacteria were harvested, and lysed by six cycles of sonication for 30 seconds in tris lysis buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 0.1% NP-40, 5mM MgCl<sub>2</sub>, 10mM 2-mercaptoethanol, 10% glycerol, protease inhibitor cocktail (Roche)). Lysates were cleared at 12'000 g for 20 min and applied to a glutathione sepharose column (GE Healthcare glutathione sepharose High Performance). Bound proteins were washed with tris wash buffer (50mM Tris-HCl pH 8.0, 100mM KCl, 0.1% NP-40, 5mM MgCl<sub>2</sub>, 10mM 2-mercaptoethanol, 10% glycerol), eluted with tris elution buffer (20mM Tris-HCl pH 8.0, 100mM KCl, 0.1% NP-40, 5mM MgCl<sub>2</sub>, 5mM 2-mercaptoethanol, 10mM reduced glutathione, 20 % glycerol) and dialysed against elution buffer without GSH. HIS-MxA and HIS-Mx1 have been previously described (28). Proteins were expressed in E.coli M15 prep4 cells with 10μM IPTG for 3 hours at 28°C. Bacteria were harvested, and lysed with two french press cycles in tris lysis buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 0.1% NP-40, 5mM MgCl<sub>2</sub>, 10mM 2-mercaptoethanol, 30mM Imidazole, 10% glycerol, protease inhibitor cocktail (Roche)). Lysates were cleared at 20'000 g for 20 min and applied to a glutathione sepharose column (GE Healthcare Glutathione sepharose High Performance). Bound proteins were washed with Tris wash buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 0.1% NP-40, 5mM MgCl<sub>2</sub>, 10mM 2-mercaptoethanol, 30mM imidazole, 10% glycerol), eluted with tris elution buffer (20mM Tris-HCl pH 8.0, 100mM KCl, 0.1% NP-40, 5mM MgCl<sub>2</sub>, 5mM 2-mercaptoethanol, 300mM imidazole, 20% glycerol) and dialysed against elution buffer without imidazole. The affinity purified proteins were analysed on a 10 SDS-polyacrylamide gel. One μg of recombinant protein were loaded per lane.

**ALPHA screen assay-** The Alpha screen assay (Perkin Elmer) was performed in a 384-well OptiPlate with 25μl reaction volume. The recombinant proteins were incubated for 2 hours at room temperature at a final concentration of 30nM each protein in Alpha screen buffer (PBS pH 7.2, 0.1% BSA), together with AlphaLISA anti-GST acceptor beads and Alpha screen Nickelate donor beads at a concentration of 20μg/ml. Interactions were analyzed on a Perkin Elmer Envision device.

**Split GFP system -** First, the coding sequences of eGFP aa158-238 including the entire multiple cloning site (MCS) of pEGFP-C1 (Clontech) was amplified by PCR and ligated into the MCS of the pCDNA3.1 (-)neo vector (Invitrogen) yielding the vector pCDNA3.1-GFP158-238. To generate the vector pCDNA3.1-GFP1-157 the coding sequence of eGFP aa158-238 in the vector pCDNA3.1-GFP158-238 was replaced by a PCR product encoding eGFP aa1-157. Subsequently the open reading frames of human MxA, MxB, UAP56 or URH49 were amplified and introduced in frame at the 3' end of the MCS of EGFP. Expression of the splitGFP fusion proteins was verified by western blot analysis using specific antibodies directed against GFP, Mx protein or UAP56 and URH49.

**Indirect immunofluorescence analysis-** A549 or 3T3 cells were grown on chamber slides for 24 hours before being transfected. Cells were fixed with 4% paraformaldehyde and mounted in DAPI containing mounting medium (Fluoromount, Southern biotech) when used with the split GFP system. Otherwise cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min. Cells were washed twice with PBS and primary and secondary antibodies were diluted in 1% BSA and incubated for 1 hour at room temperature. Cells were washed three times after each step. Anti-FLAG antibody (rabbit, 1:2000, Sigma), monoclonal mouse anti-MxA (1:20, Clone 143) were used as primary antibodies. As secondary antibodies, Alexa 488 anti-rabbit and Alexa 594 anti mouse (1:1000, Invitrogen) were used. Slides were mounted in Fluoromount mounting medium containing DAPI (Southern biotech). Samples were analyzed with a Leica TCS SP5 microscope using the LAF software. Quantification of the subcellular distribution of UAP56 or URH49 was performed using the ImageJ software.

## RESULTS

**Co-immunoprecipitation of MxA protein with UAP56 or URH49 -** Preliminary experiments aimed at testing several known NP-associated proteins for their binding capacity to Mx proteins, revealed that UAP56 but none of the other cellular proteins tested interacted with MxA (data not shown). Hence, we first analysed the interaction of human MxA with UAP56 and its paralog URH49. For this purpose, 293T cells were transfected with plasmids encoding FLAG-tagged UAP56 or URH49 in combination

with plasmids encoding MxA. UAP56 and URH49 were immunoprecipitated with an anti-FLAG antibody and the lysates were analysed by western blotting (Fig. 1A). The data show that both RNA helicases, UAP56 as well as URH49 interacted with MxA. To eliminate the possibility of a transfection artefact we also performed co-immunoprecipitations with endogenously expressed proteins. MxA expression was induced in human A549 cells by treatment with interferon- $\alpha$  for 18 h. MxA was immunoprecipitated with a monoclonal anti-MxA antibody (isotype IgG2a) and western blots with a polyclonal antibody detecting both UAP56 and URH49 were carried out to assess whether UAP56 and/or URH49 were co-precipitated. As a negative control we carried out an immunoprecipitation using a monoclonal anti-NP antibody with the IgG2a isotype as control (Fig 1B). Again the data clearly indicate that interferon- $\alpha$  induced MxA formed a complex with UAP56/URH49 (Fig. 1B).

Binding of Mx proteins to UAP56 and URH49 in vitro - To assess the relative binding capacity of MxA and Mx1 to UAP56 and URH49 we next tested these interactions in vitro employing the ALPHA Screen assay. For this purpose, recombinant GST-tagged UAP56 or URH49 and His-tagged Mx1 or MxA proteins were expressed in E.coli and affinity purified (Fig 2C). We and others have previously shown that affinity purified His-tagged Mx proteins exhibit a high intrinsic GTPase activity and antiviral activity in vitro (28,29). For measuring the interaction between Mx proteins and UAP56 or URH49 recombinant proteins (30 nM of each protein in 25  $\mu$ l reaction volume) were mixed and incubated together with fluorescent-labelled anti-GST acceptor beads and Ni-chelate donor beads resulting in a fluorescence signal only upon interaction of the bead-bound proteins (Fig. 2A). Incubation of affinity-purified GST with MxA or Mx1 resulted only in background signal. Quantification of the data revealed a slightly stronger binding of MxA and Mx1 to UAP56 than to URH49 (Fig. 2A). Analysis of the affinity purified proteins on an SDS-polyacrylamide gel revealed that a slightly larger amount of full length GST-UAP56 than GST-URH49 was present in the protein preparations (Fig. 2B) suggesting that the difference in binding was due to the difference in the amount of full length proteins. The interaction of Mx1 with UAP56 or URH49 yielded a 2-3 fold higher

signal than the interaction of MxA with the two helicases (Fig. 2A, B). This difference in signal intensity was clearly not due to differences in protein amounts used in the assay (Fig 2B). The additional 30 kDa band seen in the Mx1 protein preparation (Fig. 2B) is due to abortive translation at the N-terminus of the Mx1 coding sequence (28).

Subcellular localization of the complex formation between Mx proteins and UAP56 or URH49 - MxA exhibits a typical granular staining pattern in the cytoplasm and associates partially with the smooth endoplasmic reticulum, (30-32). UAP56 and URH49 accumulate primarily in the nucleus in RNA-splicing speckled domains and nearby nuclear regions, although for UAP56 there is also evidence for nucleo-cytoplasmic shuttling (33-35). Hence we examined in which subcellular compartment the interaction between MxA and UAP56/URH49 takes place. Mouse 3T3 cells were transfected with plasmids encoding MxA and FLAG-tagged URH49 or UAP56. As negative controls served 3T3 cells transfected with MxA protein alone. As expected, UAP56 and URH49 accumulated primarily in the nucleus forming nuclear speckles, as it has been previously described. MxA showed a clear cytoplasmic staining (Fig. 3A). Interestingly, upon coexpression of MxA with either UAP56 or URH49 we observed accumulation of UAP56 and URH49 also in the cytoplasm of cells expressing MxA (Fig. 3A) while MxA localization remained unchanged. UAP56 and URH49 were distributed throughout the whole cell. These results indicate that the observed interaction (Fig. 1 and 2) most likely takes place in the cytoplasm.

In addition we tested whether mouse Mx1 and UAP56 or URH49 would co-localize in the nucleus. Mx1 accumulates in the nucleus in distinct dots which are in close proximity to PML nuclear bodies (36,37). Immunostaining of cells co-transfected with plasmids coding for Mx1 and FLAG-tagged UAP56 or URH49 indeed revealed a pronounced co-localization of Mx1 with the two RNA helicases (Fig. 3B). Moreover, overexpression of Mx1 and the FLAG-tagged RNA helicases did not lead to leakage of these proteins into the cytoplasm (Fig 3B), indicating that the translocation of UAP56 and URH49 into the cytoplasm of MxA expressing cells is not a transfection artefact. Nevertheless, we next tested whether endogenous expression of MxA in A549 cells would lead to translocation of a fraction of

endogenously expressed UAP56 or URH49 from the nucleus into the cytoplasm (Fig. 4). For this purpose, A549 cells were treated overnight with interferon- $\alpha$  to induce expression of MxA. Immunostaining with antibodies specific for UAP56 (panel A) and URH49 (panel B) revealed that in the presence of MxA indeed a small amount of these RNA helicases could be detected in the cytoplasm while the majority of the proteins remained in the nucleus (Fig 4, panels A and B).

To verify that the translocation of UAP56 and URH49 is the result of a complex formation with MxA we made use of the so called split GFP system to further study the interaction between MxA and UAP56/URH49. To that end we adapted a system which has been previously described (38,39) fusing either the N-terminal region (amino acids 1-157) or the C-terminal region of EGFP (amino acids 158-238) to the N-termini of MxA, Mx1, UAP56 and URH49, using a 20 amino acid linker to allow for an efficient refolding of the two GFP parts upon interaction of the fusion proteins. UAP56 and URH49 have been previously described to produce homodimers (40) and MxA as well as Mx1 are able to form homodimers and higher ordered oligomeric structures [reviewed in (41)]. Hence, we used the homodimer formation of MxA, Mx1, UAP56 and URH49 proteins fused to the N-terminal and C-terminal region of GFP as positive controls (Fig. 5, panels A and B). As a negative control we co-transfected plasmids coding for human GFP1-157-MxB and human GFP158-238-MxB, which are not able to form hetero-oligomers (42). In addition co-expression of the N- and C-terminal region of the GFP protein alone yielded no fluorescence signal (Fig. 5A).

As expected we observed a speckled GFP signal predominantly in the cytoplasm (in 96% of GFP positive cells) when plasmids encoding GFP1-157-MxA and GFP158-238-MxA were co-transfected (Fig. 5A). We also observed that homo-dimerization of UAP56 or URH49 occurred primarily in the nucleus (in 92% and 92% of GFP positive cells) also showing a speckled pattern (Fig. 5A). However, co-transfection of plasmids expressing GFP1-157-MxA and GFP158-238-UAP56 or GFP158-238-URH49 resulted in GFP signals located in the cytoplasm (in 95% and 96% of GFP positive cells) (Fig. 5A). This results clearly indicate that the complex formation between MxA and the two RNA helicases occurs in the cytoplasm,

supporting our data obtained from the immunofluorescence analysis where we observed an increased translocation of UAP56 or URH49 into the cytoplasm in cells expressing MxA (see Figs. 3 and 4).

The mouse Mx1 protein accumulates in the nucleus and only exhibits antiviral activity against certain RNA viruses (all members of the orthomyxoviridae) with a replication step in this compartment (for a review see (4)). Hence, co-expression of GFP1-157-Mx1 with GFP158-238-UAP56 or GFP158-238-URH49 yielded GFP signals almost exclusively in the nucleus (in 95% and 93% of GFP positive cells) in form of distinct dots (Fig. 5B).

## DISCUSSION

Human MxA as well as mouse Mx1 protein exert a pronounced antiviral activity against IAV (4), but so far no direct interaction between Mx proteins and IAV has been demonstrated.

There is increasing evidence that the RNA helicases UAP56 and in part also URH49 are required for efficient IAV replication (18,25,27). UAP56 can bind to free NP as well as viral RNPs of IAV (17,18). UAP56 is involved in the nuclear export of several IAV mRNAs (26,27). Moreover, UAP56 is also required during IAV replication to prevent the accumulation of dsRNA in the cytoplasm of infected cells (27). For IAV so far no direct interaction between Mx proteins and viral proteins was detected and the molecular mechanism of Mx proteins against IAV remains elusive. Hence, based on our results presented here we propose that UAP56 might be the missing link between Mx proteins and the NP or vRNP of IAV. Here we show that human MxA and murine Mx1 bind to UAP56 and URH49 *in vitro* as well as *in vivo*. Intriguingly, we observed a pronounced binding of MxA to UAP56 and URH49 in co-immunoprecipitation experiments despite the fact that MxA is located in the cytoplasm and UAP56 as well as URH49 are predominantly located in the nucleus (31,33,35). Our data clearly show that the interaction of MxA with UAP56/URH49 takes place in the perinuclear region of the cytoplasm (Fig. 5A). In this context it is interesting to note that knockdown of UAP56 leads to the accumulation of dsRNA in the cytoplasm of IAV infected cells (27). So far all available evidence indicates that MxA protein exerts its antiviral function against IAV and other viruses in the cytoplasm [for a review see

(1)]. There is no experimental evidence that MxA shuttles to the nucleus. Studies aimed at elucidating the step of influenza virus replication blocked by MxA revealed that it inhibits a poorly defined step following primary transcription of viral mRNA (43). Furthermore, forced expression of MxA in the nucleus by means of a foreign nuclear translocation signal resulted in an efficient inhibition of primary transcription of IAV, indicating that the nuclear form of MxA mimics the activity of mouse Mx1 (44). Hence, if MxA were to exert its anti-influenza activity in the nucleus we would expect inhibition of primary transcription.

In addition, ectopically expressed UAP56 and URH49 showed a partial accumulation (partial retention) in the cytoplasm when co-expressed with MxA (Figs. 3 and 4). This was clearly not the case when both helicases were co-expressed with Mx1 that accumulates in the nucleus (Fig. 3). These data suggest that newly synthesized UAP56 and URH49 are either retained in the cytoplasm or are able to shuttle between the nucleus and cytoplasm. Indeed, Thomas and colleagues have now demonstrated that UAP56 and URH49 are nucleo-cytoplasmic shuttling proteins (45). In addition, two recent studies implicate UAP56 to exhibit distinct activities in the cytoplasm showing (i) that UAP56 is involved in proper translocation of mRNAs in the cytoplasm and (ii) that UAP56 exerts similar activity as eIF4A in protein translation in cardiomyocytes (34,46).

The *in vitro* binding studies revealed that Mx1 binds to UAP56 or URH49 2 to 3 fold more efficiently than MxA (Fig. 2). In this context it is interesting to note that Mx1 exhibits a more pronounced antiviral activity against IAV than MxA (28,44,47). However, it is also conceivable that for the nuclear Mx1 simply more UAP56 and URH49 are available since UAP56 and URH49 accumulate primarily in the cell nucleus. Interestingly, *in vitro* binding of MxA or Mx1 protein to UAP56 or URH49 did not require GTP-binding or GTP-hydrolysis. Addition of GTP or its non-hydrolysable analogue GTP- $\gamma$ -S had no effect on the interaction efficiency (data not shown). However, the antiviral activity of Mx proteins requires at least GTP-binding (28,29). Hence our findings indicate that binding of Mx proteins to UAP56 or URH49 appears not to represent the critical GTP-dependent step for antiviral function. The *in vitro* data further demonstrate that Mx1 binds more efficiently than MxA to UAP56 or URH49. It remains to be

determined whether this difference in binding efficiency reflects the observed differences in antiviral activities against influenza viruses. Mx1 clearly shows a more pronounced restriction of influenza virus replication than MxA (2). Influenza virus strains were recently shown to differ in their sensitivities to Mx proteins (15). Taking advantage of the viral minireplicon system Dittmann and colleagues were able to determine that the sensitivity of influenza viruses to Mx proteins segregates with NP (15). Therefore it will be interesting to test whether the different sensitivities of IAV strains to Mx proteins is reflected in different binding affinities to UAP56 and/or URH49.

Previous studies have demonstrated the MxA protein associates with viral ribonucleoprotein (vRNP) complexes of Thogotovirus (THOV) an influenza related virus and the NP of influenza virus (9,48,49). However, in none of these studies a direct interaction of MxA and NP was demonstrated. It is therefore conceivable that the observed association between the nucleocapsids of THOV and the NP of influenza virus is mediated by the cellular helicases UAP56 and/or URH49. In addition, MxA inhibits the replication of La Crosse virus by sequestering the N protein from the replication sites to perinuclear complexes (10). Hence, it will be interesting to test whether UAP56 and/or URH49 translocates together with MxA and N to these perinuclear complexes.

Taken together, we propose a new model for the antiviral activity of Mx proteins against influenza virus. In this model Mx proteins block the replication of influenza virus (and possibly also other viruses) by physically interacting with the cellular helicases UAP56 and/or URH49 required for efficient viral replication. It remains to be determined whether Mx proteins act by simply sequestering the RNA helicases from NP or vRNPs or whether Mx proteins directly interfere with the function of UAP56 and/or URH49 e.g. by inhibiting their unwinding activity. It is conceivable that nuclear forms of Mx proteins such as mouse Mx1 may interfere with the maturation and nuclear export of viral mRNA in the nucleus while cytoplasmic forms of Mx such as MxA may interfere with yet to be defined cytoplasmic activities of UAP56 and/or URH49. It also remains to be determined whether cytoplasmic forms of MxA proteins may interact with other cellular RNA helicases such as eIF4a that is involved in translation of viral mRNAs.



## REFERENCES

1. Haller, O., and Kochs, G. (2011) *J Interferon Cytokine Res* **31**, 79-87
2. Pavlovic, J., Zurcher, T., Haller, O., and Staeheli, P. (1990) *J Virol* **64**, 3370-3375
3. Frese, M., Kochs, G., Meier-Dieter, U., Siebler, J., and Haller, O. (1995) *J Virol* **69**, 3904-3909
4. Haller, O., Gao, S., von der Malsburg, A., Daumke, O., and Kochs, G. (2010) *J Biol Chem* **285**, 28419-28424
5. Gao, S., von der Malsburg, A., Paeschke, S., Behlke, J., Haller, O., Kochs, G., and Daumke, O. (2010) *Nature* **465**, 502-506
6. Di Paolo, C., Hefti, H. P., Meli, M., Landis, H., and Pavlovic, J. (1999) *J Biol Chem* **274**, 32071-32078
7. Janzen, C., Kochs, G., and Haller, O. (2000) *J Virol* **74**, 8202-8206
8. Schumacher, B., and Staeheli, P. (1998) *J Biol Chem* **273**, 28365-28370
9. Kochs, G., and Haller, O. (1999) *Proc Natl Acad Sci U S A* **96**, 2082-2086
10. Kochs, G., Janzen, C., Hohenberg, H., and Haller, O. (2002) *Proc Natl Acad Sci U S A* **99**, 3153-3158
11. Engelhardt, O. G., Ullrich, E., Kochs, G., and Haller, O. (2001) *Exp Cell Res* **271**, 286-295
12. Horisberger, M. A. (1992) *J Virol* **66**, 4705-4709
13. Huang, T., Pavlovic, J., Staeheli, P., and Krystal, M. (1992) *J Virol* **66**, 4154-4160
14. Stranden, A. M., Staeheli, P., and Pavlovic, J. (1993) *Virology* **197**, 642-651
15. Dittmann, J., Stertz, S., Grimm, D., Steel, J., Garcia-Sastre, A., Haller, O., and Kochs, G. (2008) *J Virol* **82**, 3624-3631
16. Zimmermann, P., Manz, B., Haller, O., Schwemmle, M., and Kochs, G. (2011) *J Virol*
17. Mayer, D., Molawi, K., Martinez-Sobrido, L., Ghanem, A., Thomas, S., Baginsky, S., Grossmann, J., Garcia-Sastre, A., and Schwemmle, M. (2007) *J Proteome Res* **6**, 672-682
18. Momose, F., Basler, C. F., O'Neill, R. E., Iwamatsu, A., Palese, P., and Nagata, K. (2001) *J Virol* **75**, 1899-1908
19. Fleckner, J., Zhang, M., Valcarcel, J., and Green, M. R. (1997) *Genes Dev* **11**, 1864-1872
20. Shen, H. (2009) *BMB Rep* **42**, 185-188
21. Shen, H., Zheng, X., Shen, J., Zhang, L., Zhao, R., and Green, M. R. (2008) *Genes Dev* **22**, 1796-1803
22. Luo, M. L., Zhou, Z., Magni, K., Christoforides, C., Rappsilber, J., Mann, M., and Reed, R. (2001) *Nature* **413**, 644-647
23. Kapadia, F., Pryor, A., Chang, T. H., and Johnson, L. F. (2006) *Gene* **384**, 37-44
24. Pryor, A., Tung, L., Yang, Z., Kapadia, F., Chang, T. H., and Johnson, L. F. (2004) *Nucleic Acids Res* **32**, 1857-1865
25. Kawaguchi, A., Momose, F., and Nagata, K. (2011) *J Virol* **85**, 6197-6204
26. Read, E. K., and Digard, P. (2010) *J Gen Virol* **91**, 1290-1301
27. Wisskirchen, C., Ludersdorfer, T. H., Muller, D. A., Moritz, E., and Pavlovic, J. (2011) *J Virol*
28. Pitossi, F., Blank, A., Schroder, A., Schwarz, A., Hussi, P., Schwemmle, M., Pavlovic, J., and Staeheli, P. (1993) *J Virol* **67**, 6726-6732



29. Schwemmle, M., Weining, K. C., Richter, M. F., Schumacher, B., and Staeheli, P. (1995) *Virology* **206**, 545-554
30. Accola, M. A., Huang, B., Al Masri, A., and McNiven, M. A. (2002) *J Biol Chem* **277**, 21829-21835
31. Aebi, M., Fah, J., Hurt, N., Samuel, C. E., Thomis, D., Bazzigher, L., Pavlovic, J., Haller, O., and Staeheli, P. (1989) *Mol Cell Biol* **9**, 5062-5072
32. Stertz, S., Reichelt, M., Krijnse-Locker, J., Mackenzie, J., Simpson, J. C., Haller, O., and Kochs, G. (2006) *J Interferon Cytokine Res* **26**, 650-660
33. Kota, K. P., Wagner, S. R., Huerta, E., Underwood, J. M., and Nickerson, J. A. (2008) *J Cell Sci* **121**, 1526-1537
34. Meignin, C., and Davis, I. (2008) *Dev Biol* **315**, 89-98
35. Schmidt, U., Im, K. B., Benzing, C., Janjetovic, S., Rippe, K., Lichter, P., and Wachsmuth, M. (2009) *RNA* **15**, 862-876
36. Chelbi-Alix, M. K., Pelicano, L., Quignon, F., Koken, M. H., Venturini, L., Stadler, M., Pavlovic, J., Degos, L., and de The, H. (1995) *Leukemia* **9**, 2027-2033
37. Engelhardt, O. G., Sirma, H., Pandolfi, P. P., and Haller, O. (2004) *J Gen Virol* **85**, 2315-2326
38. Ghosh, I., Hamilton, A. D., and Regan, L. (2000) *Journal of the American Chemical Society* **122**, 5658-5659
39. Hu, C. D., Chinenov, Y., and Kerppola, T. K. (2002) *Mol Cell* **9**, 789-798
40. Zhao, R., Shen, J., Green, M. R., MacMorris, M., and Blumenthal, T. (2004) *Structure* **12**, 1373-1381
41. Haller, O., and Kochs, G. (2002) *Traffic* **3**, 710-717
42. Melen, K., and Julkunen, I. (1997) *J Biol Chem* **272**, 32353-32359
43. Pavlovic, J., Haller, O., and Staeheli, P. (1992) *J Virol* **66**, 2564-2569
44. Zurcher, T., Pavlovic, J., and Staeheli, P. (1992) *EMBO J* **11**, 1657-1661
45. Thomas, M., Lischka, P., Muller, R., and Stamminger, T. (2011) *PLoS One* **6**, e22671
46. Sahni, A., Wang, N., and Alexis, J. D. (2010) *Biochem Biophys Res Commun* **393**, 106-110
47. Zurcher, T., Pavlovic, J., and Staeheli, P. (1992) *J Virol* **66**, 5059-5066
48. Kochs, G., and Haller, O. (1999) *J Biol Chem* **274**, 4370-4376
49. Turan, K., Mibayashi, M., Sugiyama, K., Saito, S., Numajiri, A., and Nagata, K. (2004) *Nucleic Acids Res* **32**, 643-652

## FOOTNOTES

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## FIGURE LEGENDS

**Fig. 1:** Human MxA interacts with cellular RNA helicases UAP56 and URH49. (A) 293T cells were transfected with FLAG-tagged UAP56 or URH49 together with MxA, cells were lysed after 48 hours and coimmunoprecipitations were performed using anti-FLAG antibodies. (B) Endogenous coimmunoprecipitations were carried out using A549 cells. For MxA expression, cells were stimulated over night with 1000U of Interferon- $\alpha$ . Immunoprecipitations were performed from whole cell lysates using a mouse monoclonal antibody against MxA (left lane). As a negative control a mouse monoclonal antibody against IAV NP was used showing no immunoprecipitation of MxA or UAP56 (right lane). (IP: immunoprecipitation, IB: immunoblot, WCL: whole cell lysate).

**Fig. 2:** In vitro interaction between MxA/Mx1 with UAP56 or URH49. (A) Affinity purified GST-UAP56 or GST-URH49 and HIS-MxA or HIS-Mx1 were mixed and assayed for (30nM of each protein) interaction in vitro using the ALPHA screen technology. As a negative control GST alone was incubated with HIS-Mx1 or HIS-MxA. The previously described interaction of UAP56 and MxA was set as a reference for the relative binding strength of the other interactions. (B) Summary of the relative interactions between the proteins in the ALPHA screen assay. (C) Purified proteins were loaded on a SDS-gel and Coomassie stained to show the purity of the protein preparations used in the ALPHA screen assays.

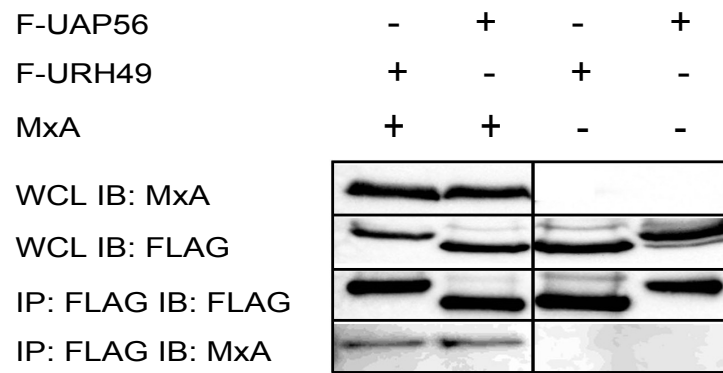
**Fig. 3:** Co-expression of MxA and UAP56 or URH49 leads to a partial retranslocation of the helicases into the cytoplasm. (A) 3T3 cells were transfected with FLAG-tagged UAP56 or URH49 with or without MxA. Cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100. Cells were stained using a polyclonal anti-FLAG antibody (1:2000) and anti-MxA antibody (1:20. Clone143). Pictures were taken with a Leica TCS-SP5 confocal microscope.

**Fig. 4:** Redistribution of UAP56 and URH49 in IFN- $\alpha$  treated cells. A549 cells were stimulated with 2000U of interferon- $\alpha$  over night and analyzed for a redistribution of UAP56 (A) or URH49 (B) as described in the legend of Fig. 3 using a Leica TCS-SP5 microscope. Unstimulated cells were used as a control for both helicases. Quantification of the UAP56 distribution (panel A) or URH49 distribution (panel B) was performed using the ImageJ software.

**Fig 5:** Subcellular localization of the complexes formed between Mx proteins and UAP56 or URH49. (A) A549 cells were transfected with GFP158-238-UAP56 or GFP158-238-URH49 and MxA (A) or GFP1-157-Mx1 (B) resulting in GFP signal only upon protein-protein interaction. As a negative control co expression of MxA and MxB were used as well as parental split GFP constructs without a protein fused to their C-terminus. Pictures were taken with a Leica TCS-SP5 confocal microscope. (C) Quantification of the subcellular distribution of the protein-protein complexes formed given in percent of cells exhibiting GFP signals in the nucleus or cytoplasm. To identify transfected cells in the negative controls cells were immunostained with antibodies directed against Mx-proteins or GFP.

Figure 1

A



B

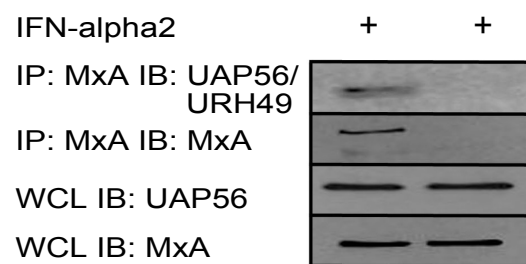
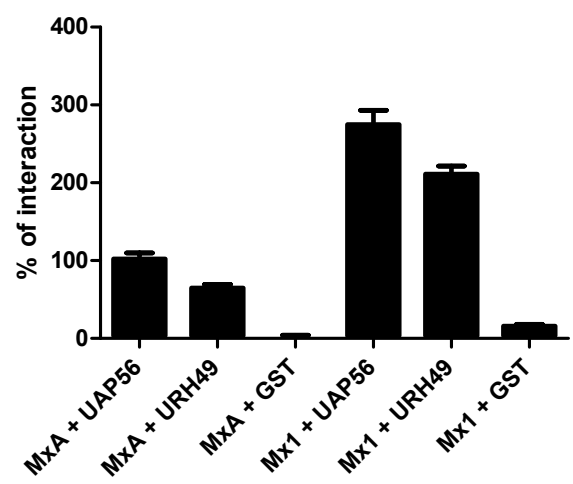


Figure 2

A



B

	% of interaction
MxA + UAP56	100.00
MxA + URH49	62.95
MxA + GST	0.29
Mx1 + UAP56	272.95
Mx1 + URH49	209.02
Mx1 + GST	13.98

C

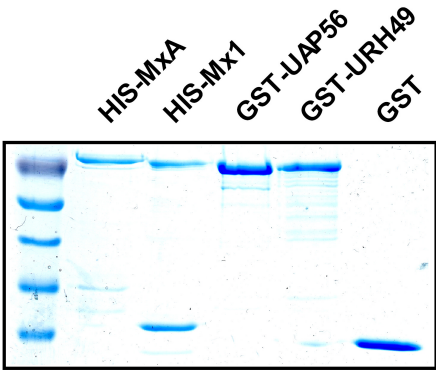


Figure 3

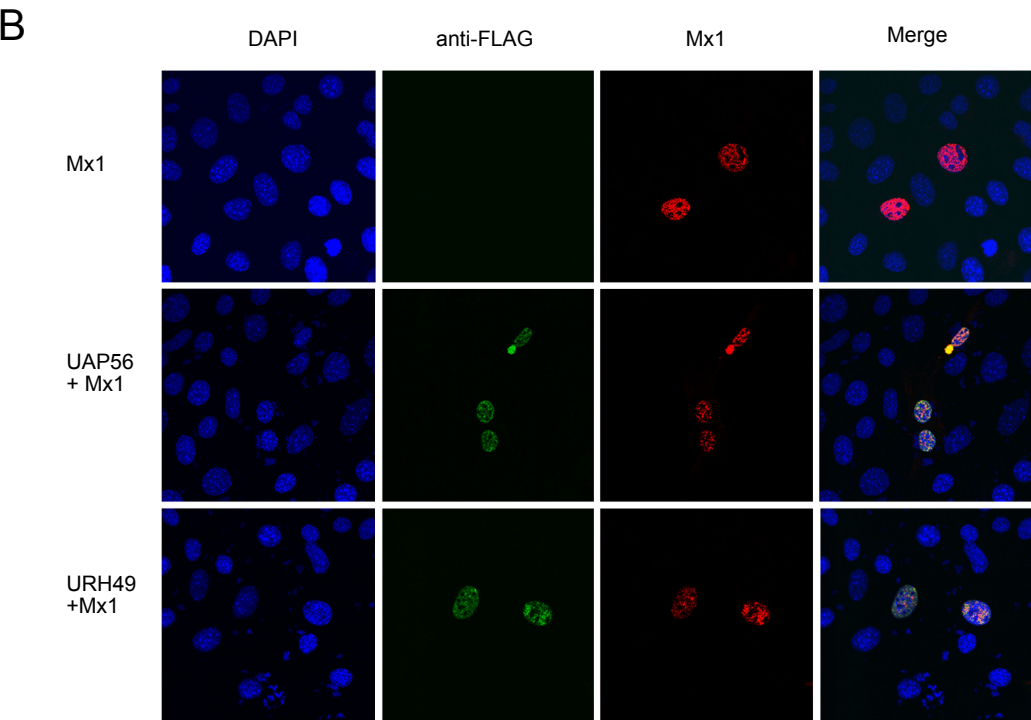
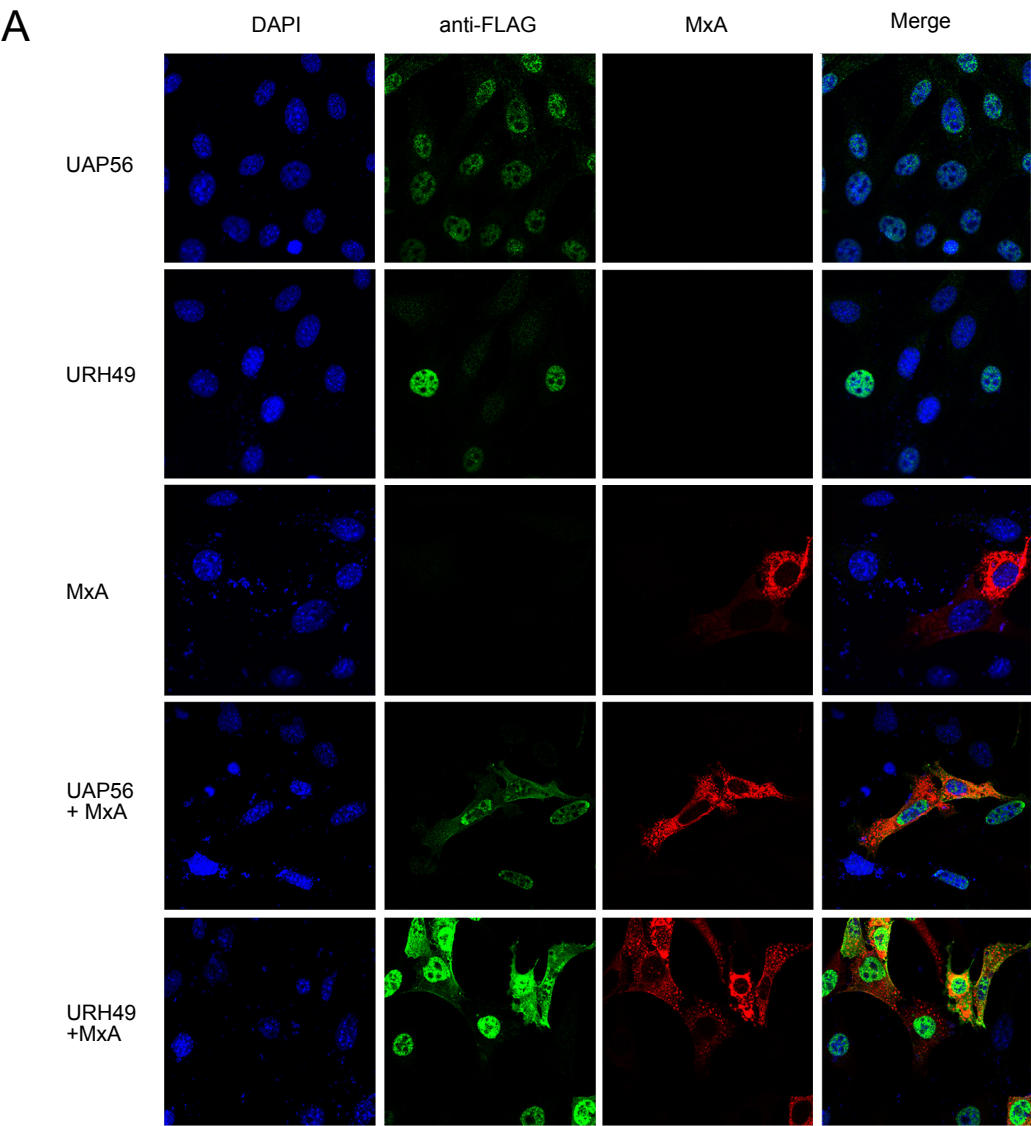
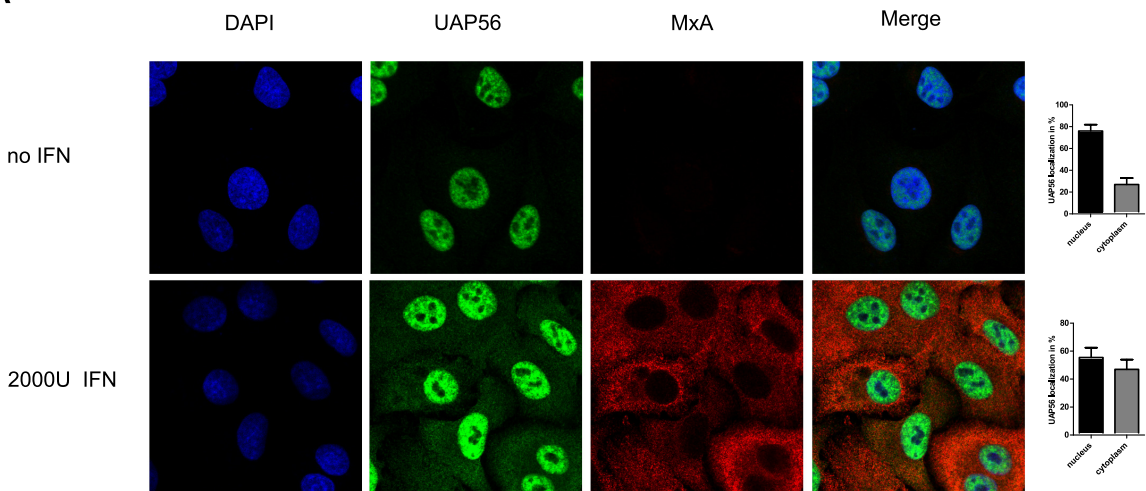


Figure 4

A



B

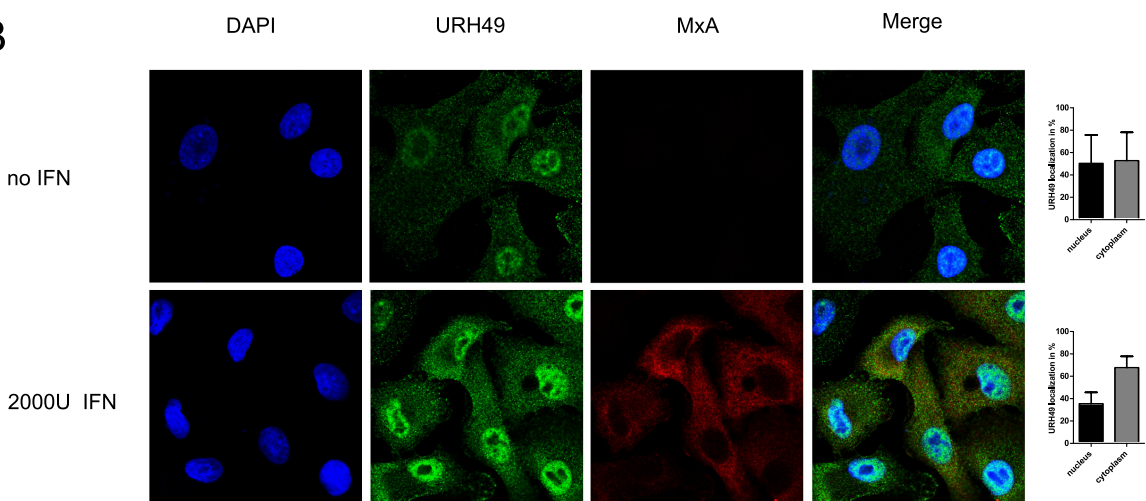
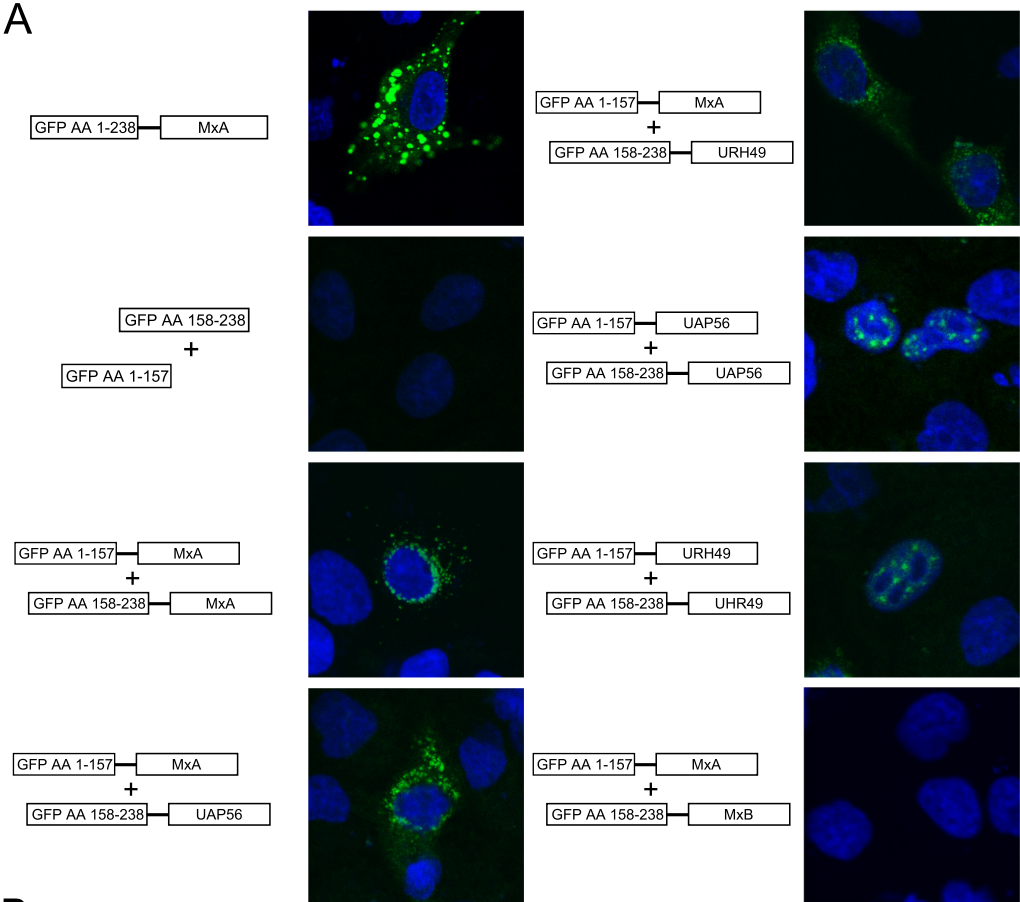
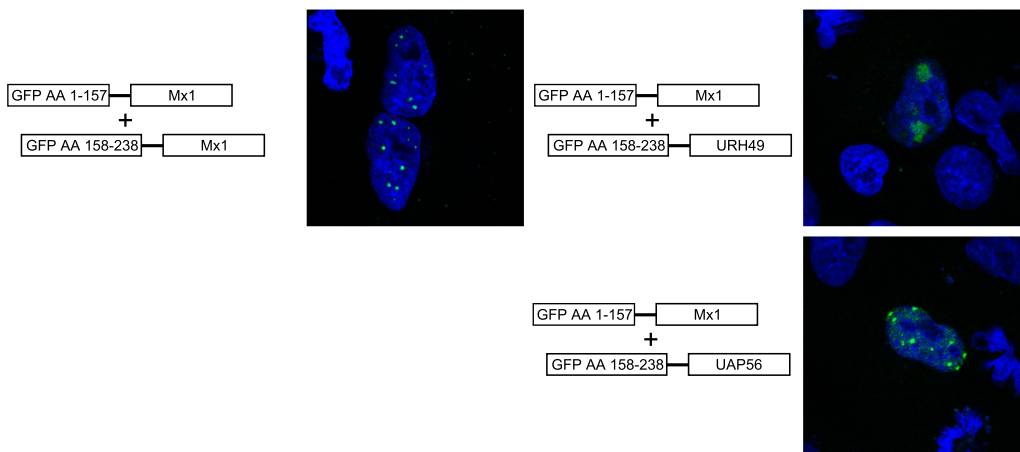


Figure 5

A



B



C

	total cells transfected	% in cytoplasm	% in nucleus
MxA x MxA	225	96	4
UAP56 x UAP56	239	8	92
URH49 x URH49	230	8	92
UAP56 x URH49	222	3	97
MxA x UAP56	233	95	5
MxA x URH49	209	96	4
MxA x MxB	241	0	0
GFP1-157 x GFP158-238	235	0	0
Mx1 x Mx1	236	6	94
Mx1 x UAP56	243	5	95
Mx1 x URH49	212	7	93